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# THE FMRFamide-LIKE NEUROPEPTIDE OF *APLYSIA* IS FMRFamide

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## ABSTRACT

The head ganglia from 350 *Aplysia brasiliana* were extracted and purified by gel (Sephadex G-15) and cation exchange (CM-Sephadex) chromatography; the fractions were examined with radioimmunoassays (RIA) for the molluscan neuropeptides, FMRFamide and $SCP_B$. Immunoreactive (ir-) FMRFamide (but not ir-$SCP_B$) coeluted with authentic FMRFamide from both chromatographic columns. The amino acid composition of the purified peptide was: Phe 2: Arg 1: Met 1. Digestion of purified ir-FMRFamide with carboxypeptidase Y indicated that the four residues were in the same sequence as occurs in FMRFamide. The dose-response curves for purified and synthetic FMRFamide on the radula protractor muscle of *Busycon contrarium* were coincident, as were their inhibition binding curves in the FMRFamide RIA. The highest concentrations of ir-FMRFamide were in the pedal and pleural ganglia; but $SCP_B$ was concentrated in the buccal ganglion. Synthetic $SCP_B$ has no effect on the radula protractor muscle of *Busycon* or the isolated heart of *Mercenaria.* In conclusion, the FMRFamide-like peptide in the gastropod *Aplysia* is FMRFamide, so this peptide has now been identified in two molluscan classes. Moreover, the proposed structural relationship between FMRFamide and $SCP_B$ is fortuitous, and these two peptides should have different physiological functions in *Aplysia.*

## INTRODUCTION

The neuropeptide FMRFamide (Phe-Met-Arg-Phe-$NH_2$) was originally isolated and identified from the ganglia of the clam *Macrocallista nimbosa* (Price and Greenberg, 1977a). More recently, a "small cardioactive peptide," $SCP_B$ (Met-Asn-Tyr-Leu-Ala-Phe-Pro-Arg-Met-$NH_2$), was isolated from the opisthobranch gastropod, *Aplysia* (Morris *et al.,* 1982). On the basis of the modest similarity in the sequences of $SCP_B$ and FMRFamide, a new peptide family including both peptides was suggested; the proposed minimum structural requirement for biological activity was Phe-A-Arg-B-$NH_2$ (where A and B are hydrophobic amino acids). However, we have found $SCP_B$ to be inactive on the *Busycon* radula protractor and on the *Mercenaria* heart, both classic FMRFamide bioassays (Price and Greenberg, 1980). Furthermore, whereas $SCB_B$ did not react in a FMRFamide radioimmunoassay (RIA), immunoreactive FMRFamide was detected in *Aplysia* ganglia in our preliminary experiments; and FMRFamide does affect neurons, gill, and the anterior gizzard of *Aplysia* (Stone and Mayeri, 1981; Weiss *et al.,* 1982; Austin *et al.,* 1983). Therefore, we suspected that *Aplysia* should possess another peptide more similar to FMRFamide than $SCP_B$. We now report that FMRFamide itself occurs in *Aplysia,* that it is chromatographically distinct from $SCP_B$, and that its distribution among the central ganglia of *Aplysia* is markedly different from that of $SCP_B$.

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## Materials and Methods

### *Purification*

The purification of Price and Greenberg (1977b) was used to isolate immunoreactive (ir-) FMRFamide from an aqueous acetone extract of head ganglia (pedal, pleural, and cerebral ganglia) from 350 *Aplysia brasiliana.* The dried extract was taken up in 2.0 ml of 0.1 *M* acetic acid and fractionated on a 2.5 × 43.2 cm Sephadex G-15 column pre-equilibrated with 0.1 *M* acetic acid, and eluted with the same. Fractions (300 drops; about 8 ml) were collected, and 2 μl aliquots of each were taken for radioimmunoassay of ir-FMRFamide and ir-$SCP_B$. Approximately 90% of the total immunoreactivity was recovered from Sephadex G-15.

The fraction from Sephadex G-15 that contained most of the ir-FMRFamide was diluted with distilled water to .05 *M,* its pH was adjusted to 5.5, and it was applied to a 0.5 × 25.0 cm CM-Sephadex cation exchange column. The column was then washed with 20 ml of 50 m*M* ammonium acetate, and the sample was eluted with a 40 ml linear gradient extending from 50 m*M* ammonium acetate (pH 6.0) to 500 m*M* ammonium acetate (pH 8.0). Fractions (1 ml) were collected, and 2 μl samples were assayed for ir-FMRFamide.

### *Amino acid composition and sequence*

The ion exchange fractions containing the majority of ir-FMRFamide were tested for purity by amino acid determination. Aliquots (200 μl) from the peak ion exchange fractions were lyophilized and hydrolyzed in 6 *M* HCl for 24 h, and their amino acid compositions were determined with an automatic analyzer (Hitachi, Model 835).

The ion exchange purified peptide was then subjected to sequential digestion with carboxypeptidase Y (Sigma). An enzyme solution (10 μl of a 1 mg/ml solution, 0.05 *M* sodium phosphate, pH 7.0) was added to one nanomole of lyophilized peptide. Digestion was stopped after 5 and 30 minutes by dilution of the sample in .025 *N* HCl, and the amino acids liberated were determined by amino acid analysis.

### *Bioassays*

Isolated *Busycon* radula protractor muscles were prepared by the method of Hill (1958) and suspended in an organ bath containing aerated, natural sea water. Tension was recorded with a force-displacement transducer connected to an inkwriting oscillograph. All drugs were added directly to the bath, and doses of FMRFamide are expressed as the final molar concentration in the bath.

### *The radioimmunoassays*

Radioimmunoassays (RIA) were used to detect ir-FMRFamide and ir-$SCP_B$ during the purification and isolation of FMRFamide. The FMRFamide RIA employed, as trace, iodinated Tyr-Gly-Gly-FMRFamide (YGGFMRFamide); the antiserum (S-253) is directed towards the COOH-terminal residues of FMRFamide. The assay is sensitive ($IC_{50}$ = .3 pmoles) and specific to the FMRFamide molecule (Price, 1983). The $SCP_B$ RIA employed iodinated $SCP_B$ as the trace, and the antiserum was provided by H. R. Morris, Imperial College of London. The $SCP_B$ assay is also sensitive ($IC_{50}$ = .5 pmoles) and specific; its cross-reactivity with FMRFamide is only .01%, where $SCP_B$ = 100%.

Each assay tube contained 100 μl of diluted antiserum, 50 μl of trace, and 50 μl of standard or unknown. Buffered saline (10 m*M* phosphate, containing 1% BSA,

0.01% merthiolate and 25 m*M* disodium EDTA; pH 7.6) was used to dilute the antiserum, trace, and standards. The tubes were incubated at 4°C for about 16 hours, and dextran-coated charcoal solution was then added to each tube to separate the bound and free antigen (1 ml of 2.5 g/l charcoal and 250 mg/l dextran in 10 m*M* phosphate buffer, pH 7.6).

## Results

When the extract of *Aplysia* ganglia was applied to Sephadex G-15, ir-FMRFamide and ir-$SCP_B$ were clearly separable (Fig. 1). Approximately 34.4 nmoles of ir-FMRFamide were recovered from fractions 20–23, whereas ir-$SCP_B$ eluted earlier; 27.2 nmoles were recovered from fraction 15–18.

Fraction 22 from Sephadex G-15 contained the majority of ir-FMRFamide, and this material was retained on the cation exchange column. A single peak of ir-FMRFamide (6.3 nmoles) eluted from the ion exchanger in fractions 21, 22, and 23 (Fig. 2). Authentic FMRFamide behaves similarly on CM-Sephadex under these same experimental conditions (Price and Greenberg, 1977b; Greenberg and Price, 1979). On HPLC ($\mu$-Bondapak $C_{18}$ column), purified ir-FMRFamide and synthetic peptide coelute at the same position in a methyl alcohol: 0.1% trifluoroacetic acid gradient (30% methyl alcohol to 50% in 20 min); synthetic $SCP_B$ elutes later.

Two amino acid analyses of the ion exchange purified peptide revealed the following compositions in nanomoles: Phe (.923; 1.194); Arg (.461; .566); and Met (.401; .408). The ratios are as expected for authentic FMRFamide (*i.e.*, about 2:1:1). Other amino

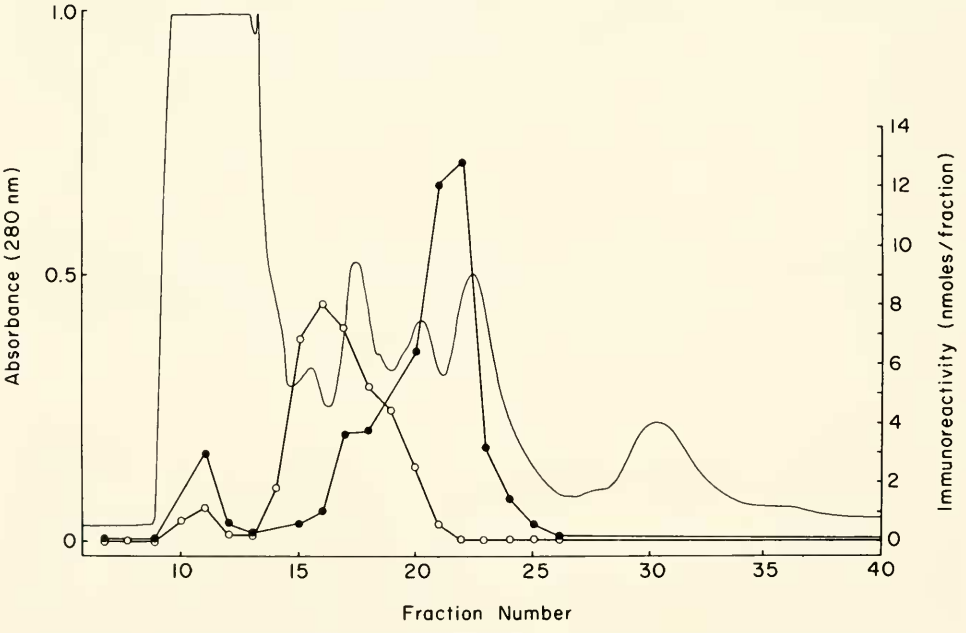


FIGURE 1. Separation of FMRFamide and $SCP_B$ from *Aplysia* head ganglia by gel chromatography. An acetone extract of the head ganglia was passed through a 2.5 × 43.5 cm Sephadex G-15 column pre-equilibrated in 0.1 *M* acetic acid, and eluted with the same. Approximately 8 ml fractions (300 drops) were collected. Immunoreactive FMRFamide (solid circles) and $SCP_B$ (open circles) were detected from 2 $\mu$l aliquots of each 8 ml fraction.

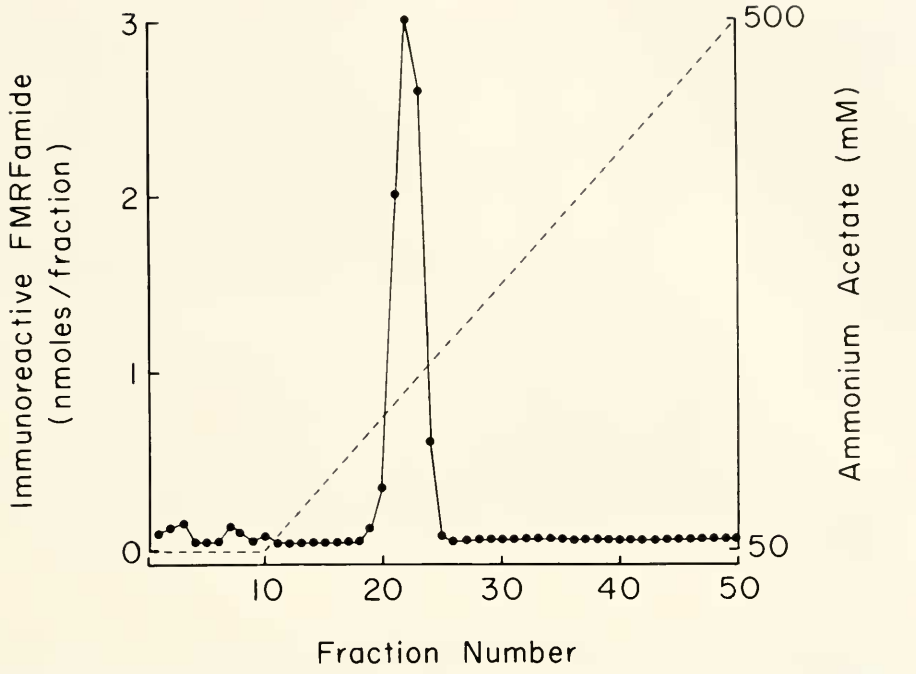


FIGURE 2. Purification of ir-FMRFamide by ion-exchange chromatography. Fraction 22 from Sephadex G-15 was applied to a CM-Sephadex cation exchange column and eluted with a 40 ml linear gradient, from 50 to 500 m*M* ammonium acetate. One ml fractions were collected, and 2 $\mu$l samples were assayed for immunoreactive FMRFamide.

acids detected included Gly (.109), Ser (.097), and Asp (.064), but they were considered to be contaminants.

Carboxypeptidase Y digestion yielded, after a 5 min digestion, only Phe (.705 nmoles) and Arg (.523 nmoles). The 30 min digestion released Phe (1.199 nmoles),

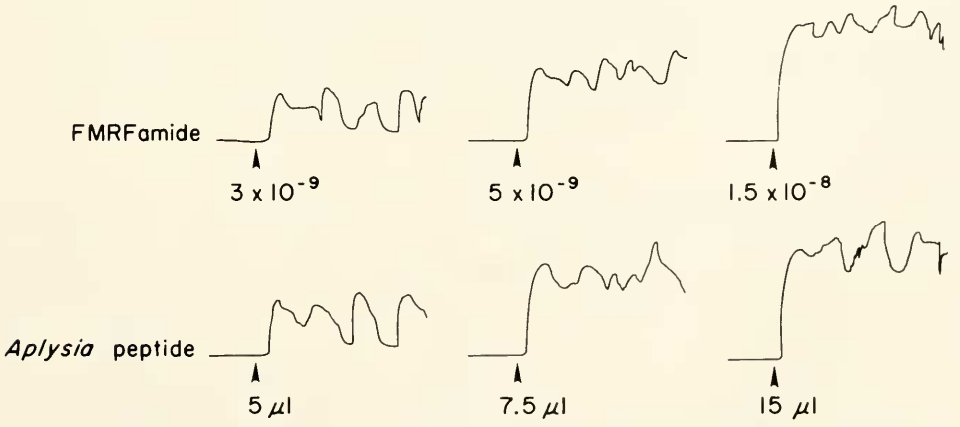


FIGURE 3. The mechanical responses of the *Busycon* radula protractor muscle to FMRFamide (upper recordings) and to aliquots of ion exchange fraction 23 (lower recordings).

Arg (.562 nmoles), and Met (.184 nmoles). Thus, the carboxypeptidase digestion indicated that the four residues were arranged in the same sequence occurring in FMRFamide.

The purified peptide from ion exchange fraction 23 was tested with the FMRFamide RIA and the *Busycon* radula protractor muscle. First, the effect of the peptide on the radula protractor muscle of *Busycon contrarium* was identical to that of FMRFamide (Fig. 3), and the dose response curves of both peptides were virtually coincident (Fig. 4). Synthetic $SCP_B$ was also tested on the radula protractor muscle of *Busycon* and the isolated heart of the clam *Mercenaria.* It was inactive on these preparations at doses as high as $10^{-5}$ *M*. Second, the isolated ir-FMRFamide peptide produced an inhibition binding curve almost coincident to that of synthetic FMRFamide in the FMRFamide-specific RIA (Fig. 5).

In summary, the quantitative estimate of peptide content in ion exchange fraction 23, as determined by RIA (2.63 nmoles) and by bioassay (3.5 nmoles), closely agrees with that found by amino acid composition (3.2 nmoles).

The amount of ir-FMRFamide and ir-$SCP_B$ in the pleural, pedal, buccal, cerebral, and abdominal ganglia were determined by RIA (Table I). Whereas the highest concentrations of ir-FMRFamide were in the pedal and pleural ganglia, $SCP_B$ was concentrated primarily in the buccal ganglia, which contains the lowest levels of FMRFamide.

## Discussion

We conclude that the FMRFamide-like peptide extracted and purified from *Aplysia* ganglia is authentic FMRFamide based on its amino acid composition, its sequence

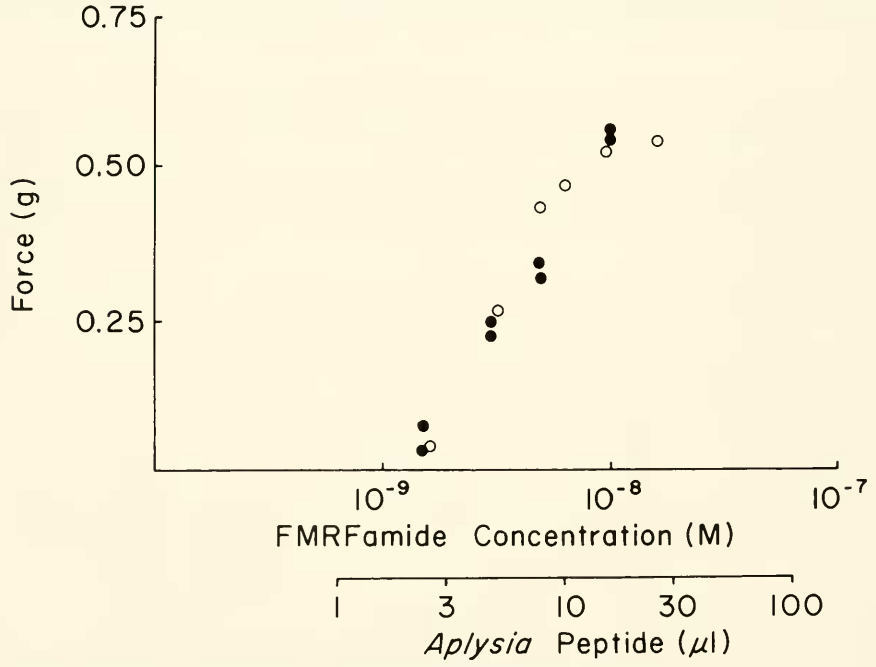


FIGURE 4. The log dose-response relations of FMRFamide (solid circles), and of aliquots of the purified *Aplysia* peptide in ion exchange fraction 23 (open circles), tested on the isolated radula protractor muscle of *Busycon contrarium.*

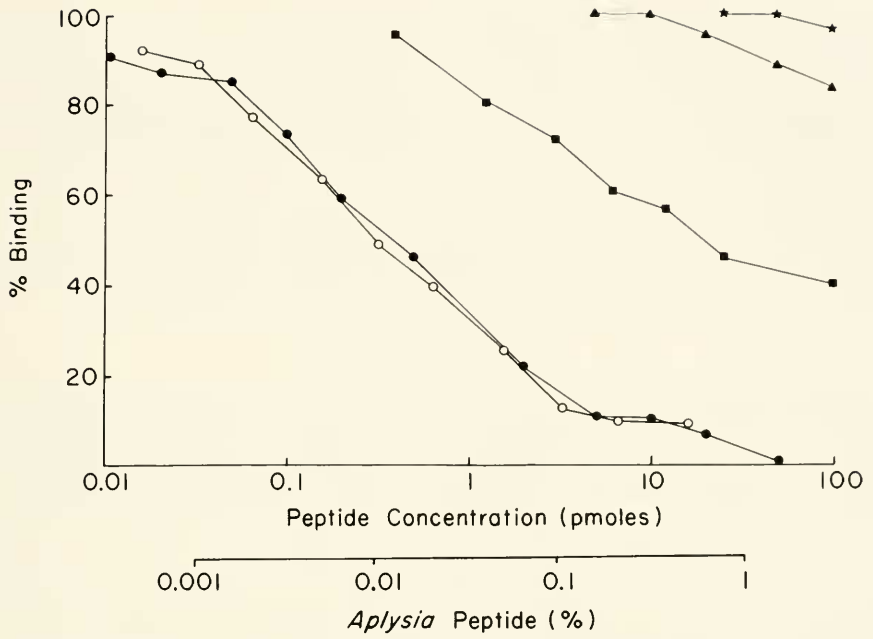


FIGURE 5. The displacement from antibody of $^{125}$I-YGGFMRFamide, by FMRFamide FMRWamide, FMRLamide, YGGFMRF, and the purified *Aplysia* peptide. The dose of *Aplysia* peptide (lower abscissa) is expressed as a percentage of the 1 ml ion exchange fraction 23.

implied by carboxypeptidase Y digestion, and its immunological and biological activity.

We have now shown that the tetrapeptide Phe-Met-Arg-Phe-$NH_2$ exists in two widely separate molluscan classes, Gastropoda and Bivalvia (Price and Greenberg, 1977a). Furthermore, the pulmonate gastropod *Helix aspersa* contains an extended peptide with a very similar COOH-terminal tetrapeptide which is active in both immunological and biological assays for FMRFamide (Price, 1982). Therefore, the FMRFamide-like family of peptides in the phylum Mollusca is characterized by activity in specific definitive bioassays; and this activity, in turn, requires a COOH-terminal tetrapeptide of relatively invariant sequence (Painter *et al.*, 1982).

Immunoreactive FMRFamide and ir-$SCP_B$ are not only chromatographically separable, they also have distinct distributions within the central ganglia of *Aplysia*. $SCP_B$

TABLE I

*The distribution of ir-FMRFamide and ir-$SCP_B$ in the central ganglia of* Aplysia

| | ir-FMRFamide | ir-$SCP_B$ |
|---|---|---|
| Pedal | 43.5 ± 10.7 | 23.1 ± 10.5 |
| Pleural | 34.3 ± 8.3 | 9.1 ± 3.0 |
| Abdominal | 18.6 ± 5.4 | 6.8 ± 3.6 |
| Cerebral | 15.7 ± 8.8 | 16.1 ± 8.7 |
| Buccal | 7.6 ± 2.5 | 75.0 ± 45.3 |

Values are given as pmoles/pair of ganglia ± standard deviation. Each value represents five separate determinations of five pairs of ganglia.

has only a slight structural resemblance to FMRFamide. In particular, its COOH-terminal tetrapeptide sequence, critical for FMRFamide-like activity, is markedly different; thus its lack of effect on both the radula protractor muscle and the clam heart was expected. Furthermore, several identified vertebrate peptides (*e.g.,* $\gamma$1 MSH and LPLRFamide) (Dockray *et al.,* 1983) are at least as similar to FMRFamide as is $SCP_B$; and $SCP_B$ is as similar to bovine pancreatic polypeptide and arginine vasopressin as it is to FMRFamide.

In conclusion, the structural similarity between $SCP_B$ and FMRFamide appears to be fortuitous; and the receptors, functions, and distribution of $SCP_B$ in *Aplysia* should be distinct from those of FMRFamide. Our findings, that the distribution and activity of the two peptides are, in fact, different, support this conclusion.

## ACKNOWLEDGMENTS

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